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13. ABSTRACT ***(Maximum 200 Words)***

Heregulin (HRG) is a growth factor that activates *erbB-2-3-4* receptors. We have generated a novel model of tumor progression from a hormone-dependent to a hormone-independent phenotype by introducing HRG into breast cancer cells. We now would like to investigate the mechanism by which HRG induces tumor progression. Our working hypothesis is that expression of HRG induces an uncontrolled mitogen-activated protein kinase (MAPK) cascade producing unbalanced growth promoting genes. **The proposed studies aim to determine whether blocking MAPK activation, cells revert to become hormone-dependent and antiestrogen responsive.**

During the first year of funding, we maintained the timeline outlined in the statement of work: a) Generated a *Ras* dominant negative (N17) regulated expression vector; b) Performed transfections into a number of MCF-7/HRG cells and isolated specific drug-resistant clones; c) Partially characterized these clones; and d) Initiated the construction of the MAPK mutant. The major findings of our work are that expression of N17 in MCF-7HRG cells results in reversion from an anchorage-independent to anchorage-dependent phenotype. Moreover, when analyzing the response to estradiol, MCF-7/HRG/N17 cells regained hormonal response to a level of the wild type MCF-7 cells. **This data demonstrates activation of the MAPK via the HRG pathway promotes an aggressive hormone-independent phenotype.**

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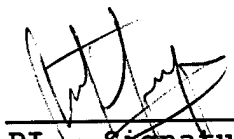

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5. INTRODUCTION

Clinical studies have shown that the *erbB-2* oncogene product, when overexpressed, correlates with tamoxifen resistance in estrogen receptor positive (ER+) breast cancer specimens. The response rate to tamoxifen in the metastatic setting varies from 50-75% in ER positive patients. In patients whose tumors overexpress *erbB-2* in the context of the ER receptor, this response rate decreases to 17%. Although the presence of ER is employed to predict the hormone dependency of a tumor, the relationship with response to endocrine therapy is not absolute (not all ER+ patients respond to endocrine therapy). Significant levels of ER have been detected in over 60% of human breast cancers, but at best only two-thirds of these ER positive tumors respond to endocrine therapy. Why this should occur is unclear. However, our experimental studies have demonstrated a relationship between the ER and *erbB-2* signaling pathways. For example, it has been shown that estradiol down regulates *erbB-2* in overexpressing cells and that ER is required for this to occur. Potentiation of breast cancer cell growth by either the ER or *erbB*-pathway may make cells less amenable to anti-proliferative strategies directed to the alternative pathway.

We have generated a unique breast cancer tumor progression model and are equipped to design and evaluate ways to revert the progressive phenotype. We showed that MCF-7 cells, which are ER-positive, progressed to a more aggressive phenotype and rendered tumorigenic and metastatic *in vivo* merely by transfecting them with HRG. It may therefore be possible to inhibit both the uncontrolled cell proliferation and the metastatic properties of breast cancer cells by blocking either HRG or the MAPK pathway. We predict that cells can bypass their normal estrogenic requirements, *if* they develop an alternative escape mechanism. One alternative pathways appears to be the *erbB*-receptors pathway. We hypothesize that there is compensation between the alternative signaling pathways. Thus, blocking one receptor pathway will result is the re-activation of the other. Clinically, that is in patients that are positive for both ER and *erbB-*, treatment with tamoxifen may result in increased proliferation through the *erbB-* signal transduction pathway. Conversely, interrupting the *erbB-* growth pathway with signaling blockers, ligand blockers or by other mechanisms may enhance proliferation through the ER system, and thereby restoring antiestrogen sensitivity.

Collectively these studies will provide a better understanding of the pathway by which cells acquire a hormone-independent phenotype and will help us to design targeted therapies for this particular population of breast cancer patients.

6. BODY

The goal of the research outlined in this proposal is to extend ongoing studies. The following experiments are designed to shed light on the biological and molecular mechanisms by which HRG mediates and/or induces cellular transformation of breast cancer cells to a more invasive and hormone-independent phenotype. The original technical objectives were as follows:

Task 1: **To determine whether changes upstream of MAPK activity play a role in HRG induction of hormone-independent breast cancer phenotype.** We will determine if Ras activation is necessary or sufficient to block HRG action. These studies will be accomplished by transfecting a dominant negative Ras mutant (N17) in a Tet-regulated expression vector into MCF-7/HRG cells. Following the transfection, drug resistant clones will be isolated and characterized. This characterization includes determining the ability of these cells to become anchorage- as well as hormone-dependent.

Task 2: **To determine the direct effect of MAPK activity, using dominant-negative MAPK mutants.** These studies will be accomplished by transfecting a dominant negative MAPK mutant in a Tet-regulated expression vector into MCF-7/HRG cells. Following the transfection, drug resistant clones will be isolated and characterized. Characterization includes determining the ability of these cells to become anchorage-dependent as well as hormone-dependent.

Task 3: **To determine the ability of specific pharmacological kinase inhibitors (PD98059-MEK1 inhibitor and PD158780- HRG/*erbB*- inhibitor) to restore hormonal responsiveness of the HRG transfected cells.** These studies will be performed *in vitro* using MCF-7/HRG cells.

STATEMENT OF WORK:

Task 1: **To determine whether changes upstream of MAPK activity play a role in HRG induction of hormone-independent breast cancer phenotype.**

ACCOMPLISHED OBJECTIVES FROM INITIAL TASKS 1 AND 2:

STATEMENT OF WORK

Months 1-8: Transfection of a dominant negative *Ras* mutant (N17); cloning of single clones expressing N17 regulated by Tetracycline

During the course of the studies, we decided to systematically perform the experiments using a regulated promoter system to drive expression of the transfected genes because:

- the transfected cells may adapt to overexpression of the mutants, and
- the generated data will show convincing specificity.

Our hypothesis was that by titrating expression from the Tet promoter, hormonal response of the dominant negative *Ras* mutant transfected cells would be regained, a direct correlation made. The levels of the *Ras**GTP will be measured when the N17 is active or inactive.

CLONING THE N17 cDNA INTO AN EXPRESSION VECTOR: We cloned the dominant negative N17 *Ras* mutant into the Tet-regulated expression vector (Invitrogen). Insert-less vectors were transfected as controls (cloning performed per manufacturer instructions).

TRANFECTIONS: Tetracycline resistant MCF-7/HRG cells were transfected by electroporation with the N17-cDNA. Resistant clones were isolated.

DETECTION OF N17-MRNA BY RNAse PROTECTION ASSAY: RNAse protection assays were used to determine if indeed N17 was expressed in the isolated clones. Five individual clones which expressed inducible levels of N17 were isolated.

Months 8-16: Biochemical and biological characterization of isolated clones: Anchorage-dependent and -independent growth assays. Assays will be performed in the presence or absence of estradiol. Biochemical characterization: measurements of *erbB*-receptor tyrosine phosphorylation and MAPK and *Ras* activity. To begin the *in vivo* experiments.

BIOLOGICAL CHARACTERIZATION OF THE N17 EXPRESSING CELLS IN VITRO USING BOTH ANCHORAGE-DEPENDENT AND -INDEPENDENT GROWTH ASSAYS:

We determined that MCF-7/HRG cells grow in an anchorage-independent fashion in the absence of estradiol. In contrast, the control cells (MCF-7/V1) grow exclusively in the presence of estradiol. Analysis of the N17-transfected MCF-7/HRG cells (MCF-7/HRG/N17) resulted in inhibition of anchorage-independent growth, as compared with control MCF-7/HRG cells (Figure 1). Moreover, when analyzing the response to estradiol, MCF-7/HRG/N17 cells regained the hormonal response to a level typical of the wild type MCF-7 cells (Figure 2), as shown in appendix I.

The ability of the dominant negative Ras mutant to abolish HRG induction of the anti-estrogen resistant phenotype will confirm the requirement of Ras in the induction of a Tam-resistant phenotype.

Task 2: **To verify the direct effect of MAPK activity, using dominant-negative MAPK mutants (ΔMAPK)**

Months 10-16: Cloning the dominant negative ΔMAPK mutant into an expression vector. Transfection of a dominant negative MAPK mutant (ΔMAPK), cloning of single clones expressing ΔMAPK regulated by Tetracycline.

CLONING THE ΔMAPK cDNA INTO AN EXPRESSION VECTOR: We cloned the dominant negative ΔMAPK mutant into the Tet-regulated expression vector (Invitrogen). Insert-less vectors were transfected as controls. (cloning performed per manufacturer instructions).

TASKS REMAINING TO BE PERFORMED FROM INITIAL TASKS 1 AND 2:

Task 1: **To determine if changes upstream of MAPK activity play a role in HRG induction of hormone-independent breast cancer phenotype by transfecting a dominant negative Ras mutant (N17) in a Tet-regulated expression vector into MCF-7/HRG cells and determine the ability of the N17-transfected cells to regain hormonal response;**

Months 12-16: Biochemical characterization: measurements of *erbB*-receptor tyrosine phosphorylation and MAPK and *Ras* activity. To begin *in vivo* experiments.

Task 2: **To verify the direct effect of MAPK activity, using dominant-negative MAPK mutants (ΔMAPK)**

Months 12-16: Transfection of a dominant negative MAPK mutant (ΔMAPK). Cloning of single clones expressing ΔMAPK regulated by Tetracycline.

TASKS REMAINING TO BE PERFORMED DURING THE NEXT YEAR FROM INITIAL STATEMENT OF WORK

Task 1: **To determine if changes upstream of MAPK activity play a role in HRG induction of hormone-independent breast cancer phenotype by transfecting a dominant negative Ras mutant (N17) in a Tet-regulated expression vector into MCF-7/HRG cells and determine the ability of the N17-transfected cells to regain hormonal response;**

Months 16-26: To continue the characterization of the N17 transfected cells in *vivo*: animal experiments will be performed in the presence or absence of estradiol, tamoxifen and IC1 182,780

Task 2: To verify the direct effect of MAPK activity, using dominant-negative MAPK mutants (ΔMAPK)

Months 16-22: Biochemical and biological characterization of isolated clones. Biochemical characterization: Measurements of *erbB*-receptor tyrosine phosphorylation, MAPK activity. Biological characterization: Anchorage-dependent and -independent growth assays. Assays will be performed in the presence or absence of estradiol. To begin the *in vivo* characterization of the transfected cells.

Months 22-36: To continue the characterization of the □MAPK transfected cells *in vivo*: animal experiments will be performed in the presence or absence of estradiol, tamoxifen and IC1 182,780

Task 3: To determine the ability of specific pharmacological kinase inhibitors (PD98059-MEK1 inhibitor and PD158780- HRG/*erbB*- inhibitor) to restore hormonal responsiveness of the HRG transfected cells.

Months 20-28: To characterize the biological activity of the specific pharmacological agents *in vitro* using all the transfected and control cell lines: Measurements of *erbB*-receptor tyrosine phosphorylation, MAPK activity. To biologically characterize the kinase inhibitors: Anchorage-dependent and -independent growth assays. Assays will be performed in the presence or absence of estradiol. To begin the *in vivo* characterization of the transfected cells.

7. KEY RESEARCH ACCOMPLISHMENTS

- Introduced the dominant negative Ras mutant into an expression vector.
- The dominant negative Ras mutant was successfully transfected into breast cancer cells that overexpress HRG.
- Isolated several individual drug resistant clones.
- Demonstrated expression of the dominant negative mutant by RNAse protection assays.
- Demonstrated that by blockage Ras activation anchorage dependent growth of breast cancer cells expressing HRG is significantly reduced.
- Demonstrated that blockage of Ras activation reverts HRG expressing cells from estrogen-independent to -dependent, therefore protecting breast cancer cells from becoming hormone-independent.
- Introduced the dominant negative ΔMAPK mutant into an expression vector.

8. REPORTABLE OUTCOMES

- Development of cell lines
- Development of animal models

9. CONCLUSIONS

The inverse correlation between HRG and ER expression in breast cancer cell lines (Table 1), prompts us to hypothesize that HRG triggers a cascade of events that lead to a hormone-independent phenotype. Therefore, we transfected HRG cDNA into an ER-positive breast cancer cell line MCF-7. The first observation after transfection was that the *erbB-* receptor signaling pathway appears constitutively activated, as shown by receptor phosphorylation and higher basal levels of MAPK activity. In addition, under estrogen-deprived conditions, the doubling time for the MCF-7/HRG cells was significantly shorter than the control cells (MCF-7/wild type or MCF-7/Vector). Moreover, estradiol (E_2) did not induce the proliferation of MCF-7/HRG cells, in contrast to control cells which were clearly stimulated. Interestingly, the MCF-7/HRG cells were unresponsive to estradiol in an anchorage-dependent fashion and also grew in an anchorage-independent fashion in the absence of Estradiol. In contrast, the control MCF-7 cells were totally dependent upon estradiol stimulation for anchorage-independent growth.MCF-7/HRG cells were hormone-independent and anti-estrogen resistant *in vivo*. The tumors developed by MCF-7/HRG cells in the presence of Tam were larger than those developed in its absence. Control cells (MCF-7/V) did not form tumors in the absence of E_2 and were tumorigenic exclusively in the presence of estradiol and sensitive to Tam, as predicted.

To decide whether the acquisition of the hormone-independent phenotype was a result of the loss of ER expression and/or ER function, we determined both the level of ER expression and the modulation of progesterone receptor (PgR) expression by estradiol. The level of ER in MCF-7/HRG was lower than the control cells and, more importantly, the level of PgR expression was not regulated by E_2. Our initial results imply that the HRG-transfected cells have lost ER function. Thus it seems that constitutive expression of HRG not only down-regulates ER but also blocks its E_2-mediated activation, abolishing the induction of PgR by $E_{estradiol}$

We hypothesized that increased activation of the *Ras-Raf-MAPK* pathway by HRG would result in transactivation of the ER receptor, thereby losing ER function. Therefore, it may be that blocking these signaling cascade cells will revert the hormone-independent phenotype induced by HRG. To validate our hypothesis we generated a *Ras* dominant negative (N17) expression vector and performed transfection. We have already determined that MCF-7/HRG cells grow in an anchorage-independent fashion in the absence of estradiol. In contrast, the control cells (MCF-7/V1) grow exclusively in the presence of estradiol. Analysis of the pool population of the N17-transfected MCF-7/HRG cells (MCF-7/HRG/N17) resulted in inhibition of anchorage-

independent growth, as contrasted with control MCF-7/HRG cells. Moreover, when analyzing the response to estradiol, MCF-7/HRG/N17 cells regained the hormonal response to a level typical of the wild type MCF-7 cells.

These studies began to elucidate the mechanism of action of HRG-mediated ER deactivation, the biological significance of blocking the MAPK pathway in restoring estrogen responsiveness and the relevance of MAPK activation in breast tumor progression. Our future studies are aimed at defining the function of these important signal transducers in breast cancer cells, especially how they control and modulate responses to growth-inducing factors. Interference with the action of specific MAPKs will provide new intervention strategies to halt breast cancer progression.

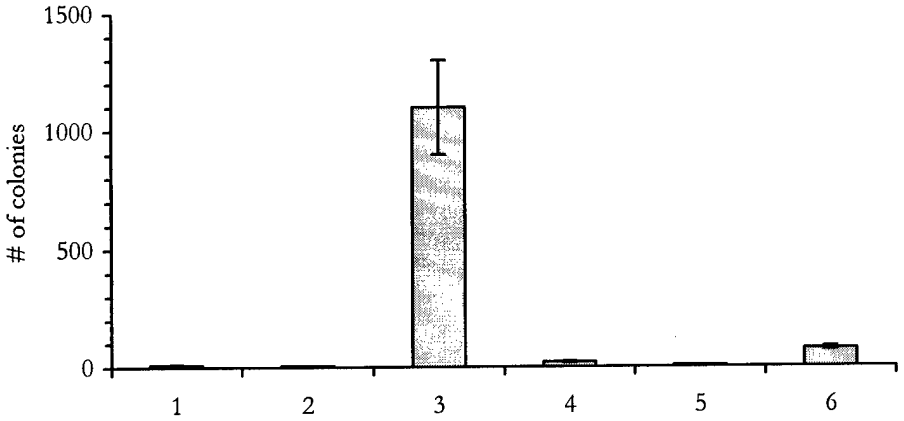
10. REFERENCES

Not Applicable

11. APPENDICES

Appendix I: Figures and Figure legends

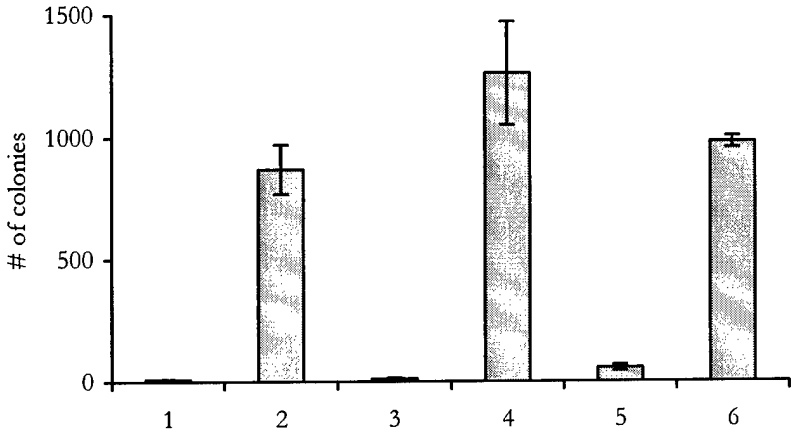
Figure 1: A dominant-negative Ras mutant (N17) blocks the anchorage-independent growth of MCF-7 cells transfected with HRG



1. MCF-7/wild type
2. MCF-7/Vector 1
3. MCF-7/HRG (T6-clone)
4. MCF-7/Vector 2
5. MCF-7/V1/N17
6. MCF-7/T6/N17

Single clones of MCF-7/HRG cells were transiently transfected with a dominant negative mutant Ras (N17). Pool population of transfected cells were grown in IMEM (phenol red-free) +5% CCS for 5 days. A bottom layer of 0.1 ml IMEM containing 0.6% agar and 10% CCS was prepared in 35 mm culture dishes. After the bottom layer solidified, cells (10,000 per dish) were added on a 0.8 ml top layer 0.4% agar, and 5% CCS. MCF-7/V1 cells denotes original vector control, MCF-7/V2 denotes control for N17 transfection (vector alone).

Figure 2: A dominant-dominant Ras mutant (N17) reverts the anchorage-independent growth of MCF-7 cells transfected with HRG



1. MCF-7/V1 Control
2. MCF-7/V1 E_2
3. MCF-7/V1 Tam
4. MCF-7/HRG Control
5. MCF-7/HRG/N17 Control
6. MCF-7/HRG/N17 E_2

Cells were grown as described in Figure 1. After the bottom layer solidified, cells (10,000 per dish) were added on a 0.8 ml top layer containing E_2 (10^{-9} M) or TAM (10^{-7} M), 0.4% agar, and 5% CCS. All samples were prepared in triplicate. The cells were incubated for approximately 12 days at 37°C.

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